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Expression of Epstein–Barr Virus Genes and of Lymphocyte Activation Molecules in Undifferentiated Nasopharyngeal Carcinomas

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Previous studies investigating the role of Epstein–Barr virus (EBV) in undifferentiated nasopharyngeal carcinoma (NPC) have been performed on extracts from biopsies. The authors analyzed expression and localization of viral gene products in 18 undifferentiated NPCs at the cellular level using immunohistology and* in situ *hybridization. All cases were EBV-positive. The small nuclear EBV-encoded RNAs, EBERs, were regularly expressed whereas the latent membrane protein, LMP1, of EBV was detectable only in four cases (22%) and the nuclear antigen 2 was not detectable. The BZLF-1 protein of EBV which disrupts viral latency, was not detectable, confirming that the virus is latent in the tumor cells. Although the expression of the CD23 antigen in transplantable NPCs has been reported, our study demonstrates that expression of this antigen in human undifferentiated NPCs is rare. In contrast, almost all cases expressed the CDw70 antigen. Since in normal tissues this antigen is present only in activated lymphoid blasts, this finding may be relevant for the differential diagnosis of undifferentiated NPCs.(Am J Pathol 1992, 140:879–887)

The Epstein–Barr virus (EBV) is associated with a number of lymphoid malignancies, e.g. Burkitt's lymphoma and Hodgkin's disease.[1–4] However, the malignancy showing the most consistent association with the virus is undifferentiated nasopharyngeal carcinoma (NPC).[5] EBV is detectable in virtually all cases of undifferentiated NPC worldwide.[1,5–7]

The expression of viral genes in EBV-immortalized lymphoblastoid cell lines (LCLs) is restricted to six nuclear antigens, EBNAs 1, 2, 3a, 3b, 3c, EBNA-leader protein, and two membrane proteins — the latent membrane protein, LMP1, and the terminal protein, TP or LMP2.[8] The pattern of EBV gene expression in EBV-associated lymphomas has been the focus of several recent studies, suggesting three different types of viral latency. In Burkitt's lymphoma, only EBNA-1 is expressed[9] in Hodgkin's disease (HD), LMP1 and presumably EBNA-1 are expressed,[3,4] and in lymphomas in immunocompromised individuals, the whole range of latent EBV proteins is detectable.[10] Two studies have demonstrated a similar pattern of EBV gene expression in undifferentiated NPCs as in HD. EBNA-1 was demonstrated in all NPC cases, and between 38% and 65% of cases showed detectable levels of LMP1 expression.[11,12] No other latent gene products and no lytic cycle antigens were detected.[11,12] The expression of the LMP1 is of particular interest in view of the known oncogenic properties of this protein. Transfection of the LMP1 gene into rodent fibroblasts induces morphologic transformation and renders the cells tumorigenic in nude mice.[13] LMP1 induces also morphologic and immunophenotypic alterations in some EBV-negative Burkitt lymphoma cell lines, most notably an up-regulation of the expression of adhesion molecules and the CD23 antigen (low affinity IgE receptor).[14] Moreover, it has been shown recently that LMP1 inhibits the differentiation of epithelial cells *in vitro* and induces morphologic transformation as well as the expression of some adhesion molecules in these cells.[15,16] Thus, LMP1 may play a role in the development of the malignant phenotype of undifferentiated NPCs. However, the expression of EBV genes in undifferentiated NPCs has only been investigated by RNA analysis or immunoblotting, meth-

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ods that rely on extracts from tumor biopsies.[11,12,17–19] Thus, the tissue distribution of the antigens could not be established and the possibility that a small proportion of tumor cells expressed additional EBV-encoded antigens at levels undetectable by these techniques cannot be ruled out. We have therefore employed a range of monoclonal antibodies against EBV-encoded proteins to investigate the expression of the EBV genome on the single-cell level. These studies were complemented by *in situ* hybridization for the detection of viral DNA and of the small EBV-encoded RNAs (EBERs) 1 and 2. These RNAs are transcribed in every known system of latent EBV-infection and the expression of the EBERs in an NPC cell line passaged in nude mice has been reported.[20–22] The expression of some lymphocyte activation-associated antigens such as the CD40 antigen in undifferentiated NPCs has been previously demonstrated.[23] Recently, the expression of the low affinity IgE-Fc receptor (CD23 antigen) in transplantable NPCs has been reported.[24] In this study, we wanted to investigate if this molecule is also detectable in human NPC biopsies. The Ki-24 (CDw70) antigen is normally present only in rare activated lymphoid cells.[25] However, it is detectable in a variety of malignant lymphomas, e.g. Hodgkin's disease (HD),[25] and recently the expression of this antigen in embryonic carcinomas has also been reported.[26] As the Ki-24 antigen is highly expressed in EBV-immortalized cell lines,[25] the possibility of its expression in EBV-associated NPCs was examined.

Materials and Methods

Tissues

Biopsy specimens of 18 undifferentiated NPCs carcinomas and one squamous cell NPC, all obtained from Chinese patients, were snap frozen in liquid nitrogen and stored at −80°C. Sections from an oral hairy leukoplakia and cytospin preparations from the X50-7 lymphoblastoid cell line were used in control experiments. For *in situ* hybridization, frozen sections and cytospin preparations were fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline (PBS, pH 7.6), dehydrated through graded ethanols and stored at −80°C. Preparations for immunohistology were air-dried overnight and then stored at −20°C.

Plasmids and Probes

The plasmid pBa-W harboring the *Bam*HI-W internal repetitive fragment of the EBV genome was provided by Dr. G. W. Bornkamm, Munich, Germany.[27] A plasmid containing a subgenomic fragment of the human papillomavirus (HPV) type 11 fragment was a gift from Dr. H. zur Hausen, Heidelberg, Germany. Total plasmid DNA was labelled with ^{35}S-dCTP (>1000 Ci/mmol, Amersham, UK) by nick translation to a specific activity of 3 to 5 × 10^8 dpm/μg. The plasmids pBSJJJ1 and pBSJJJ2 contained EBER1- and EBER2-specific fragments, respectively. These fragments were excised from the plasmids pJJJ1 and pJJJ2,[28] provided by Dr. J. Arrand, Manchester, UK and subcloned in the *Bam*HI/*Eco*RI and *Eco*RI/*Hind*III sites, respectively. ^{35}S-labelled run-off transcripts were obtained after linearization using either T3 or T7 RNA polymerases (Bethesda Research Laboratories, Gaithersburg, MD) and ^{35}S-UTP (> 1000 Ci/mmol, Amersham).[29,30] By subsequent controlled alkaline hydrolysis, the probe length was adjusted to between 100 and 200 bases. To increase the sensitivity of *in situ* hybridization, the antisense probes derived from both plasmids were mixed. Sense control probes obtained from these plasmids were also mixed.

In Situ *Hybridization*

In situ hybridization for the detection of EBV-DNA was performed as described in detail previously.[31,32] *In situ* RNA-RNA hybridization was performed according to established protocols.[30] Briefly, sections were treated with 0.2N HCl and 0.125 mg/ml pronase (Boehringer, Mannheim, Germany) and then postfixed with 4% paraformaldehyde. Subsequently, sections were acetylated with 0.1M triethanolamin, pH 8.0/0.25% (v/v) acetic anhydrid and dehydrated in graded ethanols; 25 μl hybridization mix (50% formamide/2 × SSC/10%dextran sulphate/0.5 mg/ml yeast tRNA/10mM dithiothreitol and 1–2 × 10^7 cpm/ml of labeled probe) were applied per section. Hybridization was performed for 12 to 16 hours at 50°C. Subsequently, the slides were washed in 50% formamide/1 × SSC/10mM DTT at 52°C for 4 hours, followed by digestion with 20 μg/ml RNase A for 30 minutes at 37°C and dehydration through graded ethanols.

Immobilized radioactive probes were detected by dipping the slides into a 1:2 dilution of Ilford G5 emulsion, followed by exposure at 4°C for 5 to 10 days, development, and counterstaining.

Immunohistology

Monoclonal antibodies used for immunohistologic staining are listed in Table 1. Immunohistologic staining was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique as described.[37] The

Table 1. *Monoclonal Antibodies*

Clone	Specificity	Source
BZ-1	EBV BZLF-1 protein	Dakopatts, Glostrup, DK[33]
PE-2	EBV EBNA 2	Dakopatts[10]
CS1-4	EBV LMP	Dakopatts[34]
AE-1	Cytokeratin	Boehringer Mannheim, Mannheim, Germany
Ber-EP4	Epithelial-specific glycoprotein	Dakopatts[35]
MHM6	CD23 antigen	Dakopatts[36]
Ki-24	CDw70 antigen	H. Stein, Berlin, Germany[25]

APAAP complex was provided by Dr. H. Stein, Berlin, Germany.

Results

The diagnosis of undifferentiated NPC was confirmed in all 18 cases using a mAb, AE-1, directed against cytokeratins. All cases, including the squamous cell NPC, displayed a strong and homogeneous labelling exclusively of the tumor cells, thus confirming the epithelial nature of the malignancies.

Upon *in situ* hybridization to the EBV-specific DNA probe, all undifferentiated NPCs displayed an accumulation of grains over the tumor cell population indicating the presence of EBV DNA (Figure 1a). This signal was detectable already after brief exposure (2–3 days). The signal strength observed within a given tumor was homogeneous while there was considerable variation between different cases. No signal was observed with unrelated control probes and no EBV-specific signal was observed in the squamous cell NPC as described previously.[7] Sections of nine undifferentiated NPCs were available for subsequent *in situ* hybridization analysis with RNA probes specific for the EBERs. All cases showed a strong nuclear labelling using the antisense probes while no signal was observed with the sense control probes (Figure 1 b, c). In contrast to the results obtained with *in situ* DNA-DNA hybridization, the levels of EBER expression within a given tumor showed some variability (Figure 1b).

Immunohistologic analysis of frozen sections of an oral hairy leukoplakia with the BZ-1 mAb, directed against the BZLF-1 transactivator protein of EBV, revealed a strong nuclear labeling of epithelial cells of the upper layers of this lesion as described previously[33] (Figure 2a). Expression of the BZLF-1 protein was not detectable in any of the undifferentiated NPC biopsies, indicating latent infection (Fig. 2b).

All undifferentiated NPCs showed a strong labeling with the mAb Ber-EP4, directed against an epithelial specific glycoprotein, further demonstrating the epithelial nature of the tumor cells and confirming the previously reported reactivity of this antibody with virtually all epithelial neoplasias[35] (Figure 3a); 4 of 18 undifferentiated NPCs showed a distinct labeling of tumor cells with the LMP1-specific mAbs, CS1-4. However, the tumor cells were not homogeneously labelled but showed a patchy staining pattern (Figure 3b). This observation is in agreement with staining results obtained with X50-7 lymphoblastoid cells that also showed a staining of the majority of cells with varying intensity (not shown). No staining was observed in any of the NPC cases with the EBNA2-specific mAb, PE-2, while in control experiments a clear nuclear labelling of most X50-7 cells was seen (not shown).

Using the mAb, Ki-24 (CDw70), 16 of 18 cases (89%)

Table 2. *Summary of Cases*

	ISH		IH						
Case	EBV-DNA	EBERs	LMP	EBNA 2	BZLF-1	AE-1	EP4	CDw70	CD23
1	+	+	−	−	−	+	+	+	−
2	+	+	−	−	−	+	+	+	−
3	+	nd	−	−	−	+	+	+	−
4	+	+	−	−	−	+	+	+	−
5	+	nd	−	−	−	+	+	+	−
6	+	nd	−	nd	−	+	+	+	−
7	+	+	+	nd	−	+	+	+	−
8	+	nd	+	−	−	+	+	+	−
9	+	nd	−	−	−	+	+	+	−
10	+	nd	−	−	−	+	+	+	sc +
11	+	nd	+	−	−	+	+	+	−
12	+	nd	+	−	−	+	+	−	−
13	+	+	−	−	−	+	+	+	−
14	+	+	−	−	−	+	+	+	−
15	+	nd	−	−	−	+	+	+	nd
16	+	+	−	−	−	+	+	−	−
17	+	+	−	−	−	+	+	+	−
18	+	+	−	−	−	+	+	+	−

ISH = *in situ* hybridization; IH = immunohistology; + = positive; − = negative; nd = not done; sc = scattered cells.

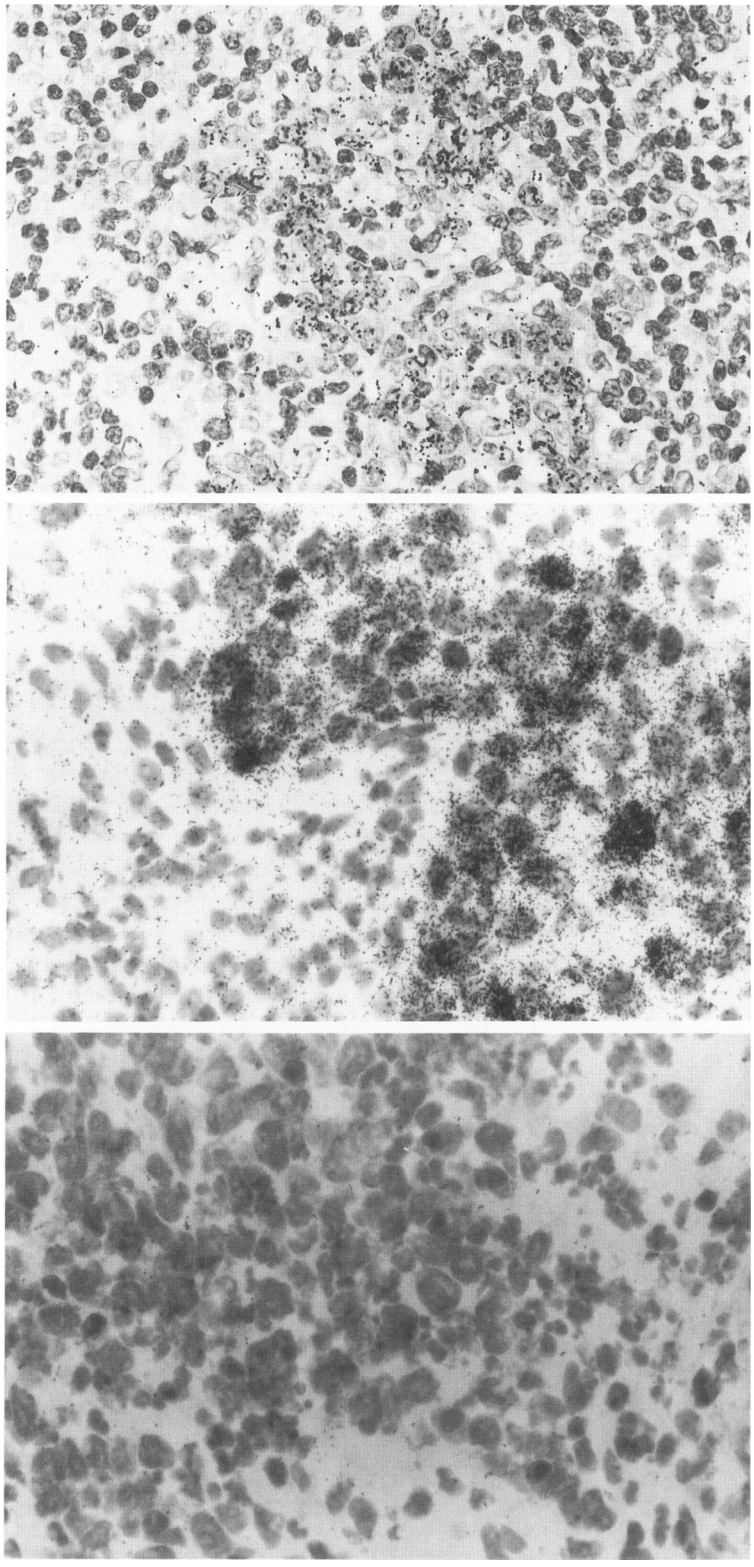


Figure 1. In situ *hybridization analysis of undifferentiated NPCs.* **A**: *Hybridization to an EBV-specific DNA probe reveals an accumulation of grains over the tumor cell nuclei, whereas only background distribution of grains is observed over reactive cells (paraffin section, 3 days exposure, H&E counterstaining, ×350)* **B**: In situ *hybridization with EBER-specific antisense RNA probes yields a labeling of tumor cell nuclei whereas* **(C)** *hybridization to the sense control probes results only in background distribution of grains (frozen sections, 5 days exposure, H&E counterstaining, ×350). Note the homogenous labeling of tumor cells after EBV-DNA hybridization, indicating a constant virus load and the marked differences in the levels of EBER expression in a given tumor.*

showed an unequivocal labeling of the tumor cell population (Figure 3c). No such labeling was observed in the squamous cell NPC. Immunostaining with the MHM6 (CD23) mAb resulted in a strong labeling of follicular dendritic reticulum cells (FDC) and in a weaker staining of follicular-mantle B lymphocytes present in some NPC biopsies (Figure 3d). In one undifferentiated NPC, a staining of a few scattered large cells was observed. These

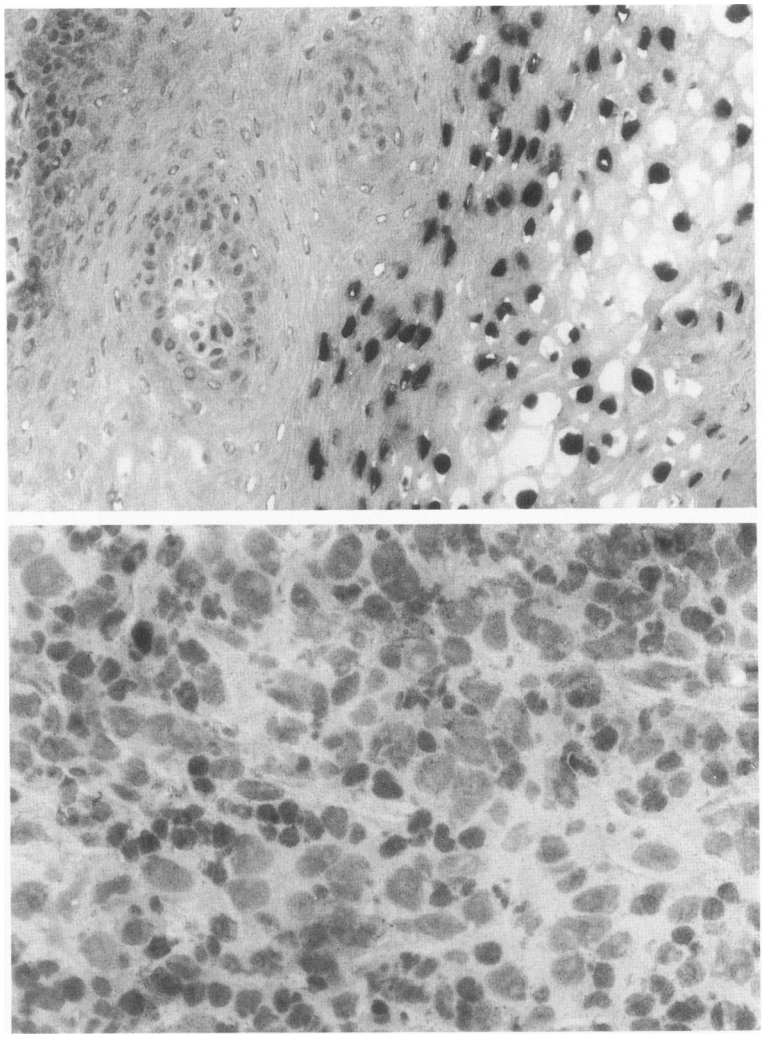


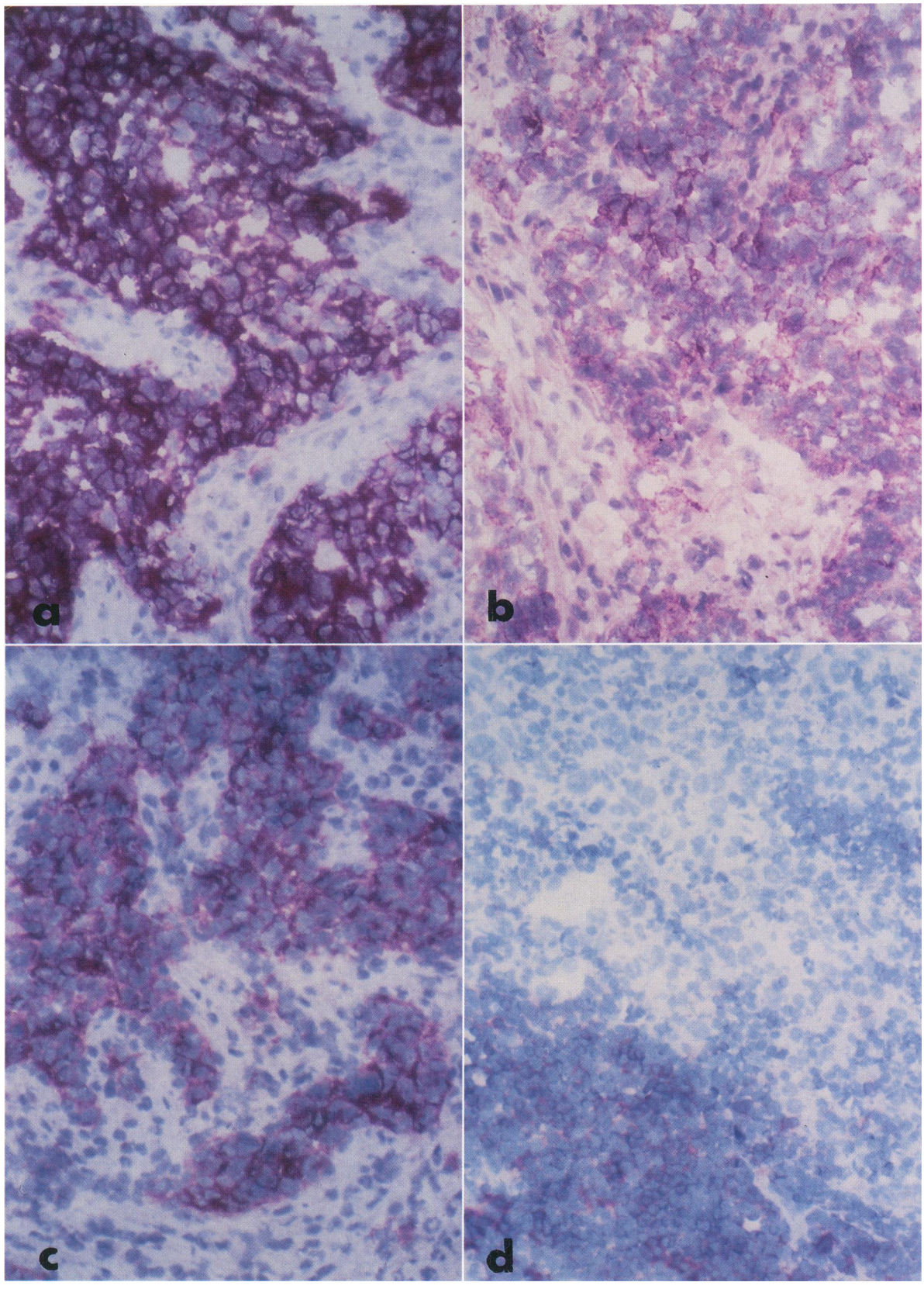
Figure 2. *Immunohistochemical staining with mAb BZ-1 reveals the expression of the BZLF-1 transactivator protein of EBV in the upper layers of an oral hairy leukoplakia (*A, *APAAP, hematoxylin counterstaining, ×225) and the absence of detectable levels of the protein from the tumor cells of an undifferentiated NPC (*B, *APAAP, hematoxylin counterstaining, ×450). Note the large pale tumor cell nuclei, whereas the smaller nuclei of infiltrating lymphocytes are stronger stained with hematoxylin.*

cells did not react with a CD21 mAb thus excluding the possibility of FDC staining. However, 16 other undifferentiated NPCs stained with the MHM6 mAb did not show any detectable expression of the CD23 antigen (Figure 3d).

Discussion

Undifferentiated NPCs are the human malignancy most consistently associated with EBV, regardless of the geographic distribution of cases.[5] However, some areas with an increased prevalence of this malignancy have been identified, most notably southern China.[5] In this study, we demonstrate the presence of EBV-DNA and the expression of EBV-encoded nuclear RNAs, EBERs, in all undifferentiated NPCs. This confirms previous studies reporting the expression of the EBERs in a transplantable NPC cell line and in human NPC biopsies.[20,38] Expression of the EBERs has been demonstrated also in Burkitt lymphoma cell lines and most recently in EBV-associated cases of Hodgkin's disease[21,22] (own unpublished observation). Thus, our results provide further evidence that the EBERs are expressed in all known lesions associated with latent EBV infection. Interestingly, although the load of viral DNA per tumor cell in a given NPC biopsy seems to be fairly constant, the signal strength obtained with the EBER-specific probes varies greatly within a given tumor. This might reflect different accessibility of the RNAs to the probes in different cells. However, it is more likely to be due to different levels of EBER expression in different tumor cells. The reason for these variations in the expression of the EBERs is not known.

A characteristic serologic feature of undifferentiated NPCs are elevated levels of IgA antibodies to EBV viral capsid antigen (VCA) and early antigen (EA).[39,40] A possible explanation for this phenomenon is virus production within the tumor cells inducing a secretory immune response. The detection of EA in extracts of NPCs has been reported.[41] However, others have demonstrated the absence of detectable amounts of lytic cycle antigens in NPCs by immunoblotting.[11,12] Thus, the possibil-


a
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ity remained that a small fraction of the tumor cells expressed late viral proteins at levels undetectable in total protein extracts. The BZLF-1 open reading frame of the EBV genome encodes an immediate early protein whose expression in B lymphocytes disrupts viral latency and induces the replicative cycle.[42] The expression of this protein precedes other lytic cycle antigens, e.g. EA and VCA, and is maintained throughout the lytic cycle.[33,42] Immunohistologic staining of frozen NPC biopsies using mAb BZ-1[33] revealed the absence of detectable levels of expression of the BZLF-1 protein whereas control sections of an oral hairy leukoplakia displayed a strong nuclear labeling of the upper epithelial cell layers. Thus, our results provide further evidence indicating latent infection in NPC tumor cells. This is also supported by the homogeneity of the EBV DNA-specific autoradiographic signal in a given tumor, indicating the absence of cells in the lytic cycle. Also, the expression of the EBERs would indicate a latent infection as these RNAs are not expressed in oral hairy leukoplakia, an epithelial lesion representing a focus of viral replication.[20,32]

Immunohistology revealed detectable levels of LMP1 expression in 4 of 18 cases (22%) whereas expression of EBNA2 was not detectable. This finding is in line with our previous immunoblotting studies demonstrating the expression of LMP1 in a proportion of cases and the absence of detectable levels of EBNA2 in undifferentiated NPCs.[11] The staining pattern within LMP1-expressing NPCs was heterogeneous showing some cell-to-cell variability. This is in line with other EBV-associated malignancies showing variation of LMP1 expression, e.g. Hodgkin's disease.[3,4] However, the frequency of LMP1 detection was considerably lower than in one previous study, in which 68% of cases showed detectable levels of LMP1 by immunoblotting.[12] The reason for this discrepancy is not clear. It may be that immunohistologic detection of LMP1 is less sensitive than immunoblotting. However, our immunohistologic results are similar to recent results obtained with reverse transcriptase polymerase chain reaction, demonstrating the detection of LMP1 mRNA in only 3 of 18 undifferentiated NPCs after one round of amplification. Subsequent amplification with nested primers led to a LMP1-specific amplification product in 15 of these 18 cases (L. Brooks et al, submitted for publication). Thus, it seems that only a minority of undifferentiated NPCs expresses significant amounts of LMP1. Recently, it has been shown that LMP1 has transforming properties not only in B lymphocytes and in rodent fibroblasts but also in epithelial cells transfected with the LMP1 gene.[13,15,16] Although these previous studies would suggest that LMP1-expression is important in the pathogenesis of undifferentiated NPC, the current results indicate that continuous expression of this protein is not required for the maintenance of the malignant phenotype. Previous studies have reported the absence of EBNA2 expression from undifferentiated NPCs as detected by immunoblotting.[11,12] Since this technique is based on the extraction of proteins from tissues, the possibility remained that occasional tumor cells expressed EBNA2 at levels undetectable in total protein extracts. We provide further morphologic evidence demonstrating the absence of EBNA2 expression from undifferentiated NPCs at the single cell level.

The expression of the low affinity IgE receptor (CD23 antigen)[43] in transplantable NPC tumors has been reported previously and a possible role for this molecule in the pathogenesis of NPCs has been proposed.[24] However, using a well-characterized mAb, MHM6, in immunohistologic studies, we found no convincing evidence for an expression of this antigen in human NPC biopsies. Only one of 17 cases stained with this antibody revealing a staining of a few scattered cells. Although these cells did not react with a CD21 mAb and thus were probably not FDCs, a nonspecific staining cannot be ruled out entirely. An explanation for the difference between the previously reported results[24] and our data may lie in the different mAbs used in the studies. However, we have previously observed negative staining results on undifferentiated NPC biopsies also with two other CD23 mAbs (unpublished observation). Thus, our results would suggest that expression of the CD23 antigen is at best rare and is no characteristic feature of undifferentiated NPCs.

The expression of the Ki-24 (CDw70) antigen in undifferentiated NPCs is an unexpected finding. The CDw70 antigen is a lymphocyte activation-associated molecule.[25] *In vitro,* the expression of this antigen in lymphoid cells is most efficiently induced by some viruses, e.g. EBV or HTLV-1.[25] In normal tissues, the expression of the CDw70 molecule is restricted to a few lymphoid blasts. The Ki-24 antigen is expressed in a variety of malignant lymphomas, most notably in the tumor cells of Hodgkin's disease.[25] The strong association of EBV with HD has only recently been disclosed.[2–4] Outside the lymphatic tissue, only embryonic carcinomas have been shown to express this antigen.[26] Thus, it is tempting to speculate that the expression of the CDw70 antigen in undifferentiated NPCs is in some way related to the presence and

Figure 3. *Immunohistochemical staining of undifferentiated NPCs* (a) *with mAb Ber-EP4 shows strong labelling of tumor cells indicating their epithelial nature,* (b) *with the mAbs CS1-4 demonstrates the expression of LMP1 in the tumor cells,* (c) *with mAb Ki-24 shows expression of the CDw70 lymphocyte activation antigen in the tumor cells, and* (d) *with the mAb MHM6 demonstrates staining of follicular dendritic reticulum cells and follicle mantle lymphocytes but absence of detectable levels of CD23 antigen from the tumor cells (APAAP, hematoxylin counterstaining, magnification, ×175).*

expression of the EBV genome in the tumor cells. This hypothesis requires further investigation. Our results demonstrate that the Ki-24 molecule is expressed in almost all undifferentiated NPCs. Thus, the presence of this antigen may provide a useful marker for the differential diagnosis of these tumors. On the other hand, our results show that expression of the Ki-24 antigen in an undifferentiated tumor does not necessarily indicate its lymphoid origin.

The association of undifferentiated NPC with EBV is well established.[5–7] However, the precise role of the virus in the oncogenic process remains unclear. The expression of EBNA1 and the EBERs in all cases and of LMP1 in a proportion of cases,[11,12] together with the monoclonality of resident EBV genomes[44] indicate that the virus is not merely a silent passenger in the tumor cells. The small proportion of EBV-infected individuals who develop undifferentiated NPC and the long interval between primary infection and tumor development point to a multistep process requiring a number of distinct events. Whether EBV infection of epithelial cells is an early event in this process is difficult to determine since premalignant lesions of the nasopharynx have not been defined. However, *in situ* hybridization for the detection of EBV DNA and EBERs together with the immunohistochemical detection of differentially expressed antigens such as the Ki-24 (CDw70) molecule may help in defining precursor lesions of undifferentiated NPCs and may contribute to our understanding of the pathogenesis of this tumor.

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